The effect of wash water chlorine content on the pinking discolouration of fresh cut Iceberg lettuce.

Keywords

Chlorine, Pinking, Discolouration, Cut-edge, Lettuce

Abstract

Iceberg lettuce is subject to pinking discolouration along cut edges during processing. Passive and active modified atmosphere (MA) packaging with very low steady state oxygen (typically < 0.2%), achieved with nitrogen flushing, limit this effect, however the industry still suffers instances of pinking. Typically, bags of processed lettuce with over 5% pinking by leaf area are rejected by the food industry. Rejections during quality assessment by suppliers, retailers and the householder, contribute to the total estimated 47% of bagged salad that is wasted across the processor to consumer supply chain in the UK. The causes of the discolouration involve complex biochemical pathways. Breeding for varieties with a reduced pinking response requires extensive resources and does not provide a short-term solution. Treatments to reduce the response, e.g., inhibitor additives and heat shock treatment, typically involve additional costs and extensions to the processing line. Minimal processors require a low-cost method to reduce pinking that can be easily incorporated into current practises. Chlorine is already used in the processing line as a sanitiser and has been previously proposed to reduce pinking. This study examined the effects of applying a range of chlorine concentrations to wash water, ≈1 mg L^{-1}, 50 mg L^{-1}, 100 mg L^{-1} and 200 mg L^{-1} total chlorine content, to reduce cut edge pinking. In the first part of this study lettuce was packaged in air and observed beyond the normal commercial shelf life period. For overall pinking score there was a clear U-shaped response to chorine concentration, with minimum overall pinking score for concentrations close to 100 mg L^{-1} chlorine.

In a second trial, using controlled atmosphere chambers, samples stored in air were compared to those exposed to an initial oxygen content of 3% and 6%, followed by changes to the atmospheric concentrations to simulate the conditions to which cut lettuce is exposed within MA bags. Pinking was monitored at days 4 and 7. There was no pinking for lettuce packaged in bags with an initial oxygen concentration of 3%. For overall pinking score a clear chlorine concentration effect was apparent for lettuce stored in air and at an initial oxygen concentration of 6%. In this study the effect was linear with lowest pinking scores for lettuce washed at the highest chlorine concentration of 200 mg L-1.

Keywords: pinking, discolouration, lettuce, modified atmosphere, chlorine

Introduction

Pre-prepared salads have become an increasingly valuable sector in both Europe and the United States over the past decade. From 2008 to 2018 spending on chilled prepared leafy salads and vegetables doubled in the UK reaching over £1.1 billion (Wunsch, 2020). In the US the sector was forecast to reach $7 billion by 2018 (Packaged Facts, 2014). Minimally processing of lettuce (*Lactuca sativa*) often involves cutting practices that can induce discolouration across the tissue edges (Saltveit, 2018), especially in the *L. sativa* varieties such as iceberg which dominate this sector of the food service industry, and which notably show ‘pinking’ as opposed to ‘browning’ (Hilton et al., 2009). This ‘pinking’ is considered aesthetically unattractive and can appear within a few hours after cutting when cut lettuce is exposed to air. The reactions leading to discolouration depend on oxygen. Consequently one important commercial practice to preserve quality is to pack cut lettuce in modified atmosphere packaging, and usually with initial nitrogen flushing to decrease the oxygen concentration. With correct packing the oxygen concentration should be very low (typically less than 0.5%) within 24 hours. In ideal supply chain conditions, i.e. packaged in modified

atmosphere and stored at 5°C, a shelf life of between 6 and 7 days is to be expected, but even under these conditions pinking has been reported by day 3. The condition is believed to contribute to the high level of bagged lettuce discarded in UK households, reported to have reached 37,000 tonnes in 2009 (WRAP, 2009), even when the additional unaccounted volume discarded by food service industry clients is not considered. The discolouration, both pinking and browning, occurs when tissue is wounded, exacerbated by stresses due to microbial contamination and associated decay. Stress stimulates an increase in the activity of phenylalanine ammonia-lyase (PAL) and the synthesis of phenolic compounds including o-diphenols such as caffeic acid and chlorogenic acid. These compounds may be oxidised by polyphenol oxidase (PPO) released from the plastids due to wounding or the subsequent tissue senescence. Depending on the interplay between various biochemical pathways, either brown or pink pigments may subsequently be formed (Toivonen and Brummell, 2008; Saltveit, 2018). Breeding for lettuce varieties with a reduced propensity for pinking will not be an expedient path to solve the problem, and so alternative methods to inhibit cut edge pinking that are both financially and logistically viable are being sought by the processing industry.

Chemical treatments have been applied to minimally processed produce, typically to control decay and inhibit microbial growth, namely chlorine, and certain organic acid solutions (Rico *et al.*, 2007). In the UK, the maximum total chlorine content, i.e. free (actively available) chlorine + combined chlorine, is recommended at <100 mg L^{-1}, before it is rinsed with potable water (Chilled Food Association Ltd, 2005). In practice, chlorine content managed manually may vary from 20 to 80 mg L^{-1} free chlorine. Such processing treatments have also been used to reduce cut-edge discolouration. Both sulphur dioxide and chlorine have been reported to reduce discolouration in cut edge lettuce (Bolin et al., 1977). Further evidence of the inhibition of browning in green peas, apples and potatoes using various hypochlorous acid

solutions were presented by Brecht et al. (1993). These authors later went on to state that browning in snap beans was significantly reduced using sodium hypochlorite concentrations of 175 mg L^{-1} at both 25 °C and 5 °C (Brecht, 1995). However, little further research was carried out to support these claims, until 2002, when Fukumoto et al. published results of their investigations into the relationship between chlorine and heat stress to reduce pinking. The study included a comparative analysis of the impact of chlorine at a 4 °C wash temperature (Fukumoto et al., 2002). It was demonstrated that pinking was less prevalent when lettuce was washed in a total chlorine content of 100 mg L^{-1} compared to water washed controls. However, no further investigation has been carried out to determine the potential impact of changes to chlorine concentration on pinking of cut edge lettuce tissue washed and processed at 5 °C. This is the standard commercial storage temperature, and a wash temperature within quality control remits. If this process can be optimised to reduce pinking, this practice would not involve additional features to a production line and would therefore be of great commercial and environmental value.

A study was carried out to identify the impact of a range of chlorine treatments on the pinking development of cut Iceberg lettuce. Minimally processed lettuce was washed in chlorine at different concentrations and then stored at 5°C in air to promote discolouration of the cut edge. Respiration rates, discolouration intensity and incidence were recorded at days 5, 7 and 11. In a second trial, using controlled atmosphere chambers, samples stored in air were compared to those exposed to an initial oxygen content of 3% and 6%, followed by changes to the atmospheric concentrations to simulate the conditions to which cut lettuce is exposed within MA bags. This simulation method provided the opportunity to compare the effect of a range of pre-packing treatments on MA packed cut lettuce. This would have been logistically very difficult within the commercial packing line.

Methodology

Study A:

Wash treatments were carried out in 10 L containers containing tap water (1 mg L^{-1}chlorine approximate), and 50 mg L^{-1}, 100 mg $L^{-1}$and 200 mg L^{-1}chlorinated water using Evans Effervescent Chlorine Tabs™ (Troclosene Sodium = Sodium dichloroisocyanurate $C_3Cl_2N_3NaO_3$) 30 – 60%, Adipic acid 20 – 25%, Sodium Carbonate 3-5%) with pH recorded at 7.4, 7.7 and 8.2 respectively. Supermarket bought iceberg lettuce was cut into 8 mm wide strips and sanitized in the chlorinated water by manually mixing for two minutes. Lettuce was spun dry. Three replicates, weighing 400 g each (+/-5%) per treatment were placed in air in air-tight sealed plastic boxes and stored at 5°C. Rates of respiration were determined by periodic measurements of the oxygen and carbon dioxide concentrations in the box using a Dual Gas Analyser GCS250. Pinking assessments were carried out at days 5, 7 and 11 days after processing. Pinking assessments were carried out using the rankings for pinking intensity and incidence developed for commercial quality assessments, as described in Table 1. Total Pinking Score = Incidence * Intensity.

Study B:

Wash treatments were carried out in 10 L containers containing tap water (1 mg L^{-1} chlorine approximate), and 50 mg L^{-1}, 100 mg L^{-1} and 200 mg L^{-1} chlorinated water by dilution of Fisher Chemical™ sodium hypochlorite solution (5.65-6%). pH was recorded as 7.5. 8.2 and 9.6 respectively. Lettuce was processed as in Study A before 18 samples for each treatment were placed in unsealed bags and divided between three SafePod™ controlled atmosphere chambers (Storage Control Systems Ltd, Paddock Wood, UK), relative humidity near 99%. The concentrations of oxygen and carbon dioxide were controlled over time to follow the profiles shown in Figure 1, which simulate the profile previously measured for bagged lettuce packed with nitrogen flushing to give an initial oxygen concentration of 3% and 6%

respectively. The third chamber was provided with air throughout. All processing and storage were carried out at 5°C. Three samples were removed for each treatment from each chamber for pinking assessment 4 and 7 days after processing, using the same protocol as for Study A. Statistical analysis was carried out using Microsoft Excel, Version 1710 , Genstat 18th edition and R version 4.0.2. For Study A, multiple regression was used to find the best fit quadratic for a U shape curve for overall pinking against chlorine dose for each time point. For Study B, multiple regression was used to find the best fit for a straight line for overall pinking against chlorine dose for each atmosphere treatment and each time point. Models were tested by Anova (results presented in the figure legends).

Results

For lettuce stored in air, higher levels of pinking, in terms of both incidence score and intensity score were consistently observed in lettuce washed in tap water compared to lettuce washed in the other three chlorinated wash treatments (Figure 2A, B). The lowest scores were seen in lettuce washed in 100 mg L^{-1} chlorine.

For pinking incidence, scores ranged between 2.0 and 2.3, at day 3 (Figure 2A), and increased notably between days 3 and 7. The lowest pinking incidence score by the end of shelf life, day 7, was 2 and was observed in the lettuce washed 100 mg L^{-1} chlorinated water. The highest scores were reached at day 11 in lettuce washed in the lowest chlorine wash of $\approx$1 mg L^{-1} (tap water).

Pinking intensity scores increased over time as expected, but without great difference between chlorine concentrations (Figure 2B), with scores at each timepoint lying within a range of one or less, and all treatments showing the same intensity of pinking by the end of the trial. This may be accounted for by the smaller range (0 to 4, as opposed to 0 to 5) in pinking incidence rankings.

For overall pinking score there was a clear U-shaped response to chorine concentration; multiple regression with quadratic curve indicated a chlorine concentration effect with minimum overall pinking score for concentrations close to 100 mg L^{-1} chlorine (Figure 2C)

In order to understand the mechanism by which chlorine treatment reduced pinking, respiration rate was used as an indicator of stress (Figure 3). Although there was a general trend with increased chlorine concentration corresponding to an increase in respiration, the range in respiration rates was small from 5.4 to 6.7 ml/Kg -h three days after processing, and 2.2 to 3.2 eleven days after processing. These figures are not indicative of highly stressed lettuce and no statistically significant difference between the treatments was found.

Study B was carried out to determine the effect of chlorine wash on pinking under conditions to simulate MA packaging. For the control, in which lettuce was stored in air, in agreement with Study A pinking was notably higher when lettuce was washed in lower concentrations of chlorine (Figure 4). As for Study A pinking incidence was affected more strongly than pinking intensity by chlorine concentration. No pinking was exhibited when lettuce was stored in conditions to simulate MA packaging with an initial oxygen concentration of 3%. This is as expected, under conditions where oxygen would deplete rapidly so that oxygen was no longer available for the oxidative reaction required to initiate the precursors to pigmentation, and as such no pinking was seen at day 4, or day 7 (equivalent to the end of commercial shelf-life). For the simulation with initial oxygen concentration of 6%, pinking only appeared on day 4 for those samples that had been washed in the lowest level of chlorine, whereas by day 7 a graduated response was observed between chlorine content of wash water and pinking, with only lettuce washed at the highest level exhibiting no pinking at all.

For overall pinking score a clear chlorine concentration effect was apparent for lettuce stored in air and at an initial oxygen concentration of 6%. In this study the effect was linear with lowest pinking scores for lettuce washed at the highest chlorine concentration of 200 mg L-1 chlorine.

Discussion

It was hypothesised that increased chlorine levels may inhibit pinking in bagged lettuce in one of two ways. Firstly, the breakdown of essential fatty acids in tissues by the chlorine could stimulate stress signalling. The consequential transduction pathways responsible for an increase in respiration rates would result in less oxygen available in the modified atmosphere (MA) bag, inhibiting the oxidation reaction. Alternatively, there may be direct inhibition of the PPO enzyme by chlorine. (Lu, Luo and Feng, 2006) found a similar inhibitory effect on PPO in apples exposed to sodium chlorite, which shares similar oxidising properties to sodium hypochlorite, and resulted in reduced browning. The group found that preincubation of PPO with 8 mM sodium chlorite for 8 min caused a total 46% activity reduction compared to noninhibited control.

The measurements of respiration rates gave no indication that chlorine wash induced stress in the cut lettuce. The hypothesis that pinking is inhibited due to the toxicity of chlorine to the PPO enzyme is supported by the reduction in pinking with increasing chlorine concentration. The decrease in effect at the highest chlorine concentration for Study A may indicate greater damaging effect of chlorine on tissues, prompting decompartmentalization that enables further pinking again. This can be seen in Study A, where we witnessed a greater pinking at 200 mg L^{-1} than 50 mg L^{-1} by day 7. This is also supported by Brecht et al, (1993), which also attribute their findings to tissue damage. They observed, at a neutral pH, that apples showed

less discolouration when treated with sodium hypochlorite at 17.5 mg L^{-1}, than at 140 mg L^{-1}. However, this appears to be commodity dependent, with potato treatment with sodium hypochlorite proving more effective at reducing browning at the higher chlorine concentration. The action of sodium hypochlorite depends on pH. When the pH is kept high enough chlorine is slowly liberated. Brecht *et al's* results also, show a difference when the pH of the solutions was adjusted, again different results, with potato discolouration at its least at the lower sodium hypochlorite concentration and a lower pH of 4, and apple at its least discoloured at pH 7 at 140 mg L^{-1}. The different relationship between pinking and chlorine concentration in the two studies presented here; U-shaped with a minimum of 100 mg L-1 chlorine for Study A, but linear for Study B may be due to difference in pH, with the lower pH in study A resulting in a higher ratio of hypochlorous acid to hypochlorite.

The exact mechanism for a relative decrease in pinking throughout shelf life that arose in lettuce washed in a higher total chlorine is not known. The antimicrobial effect is caused by irreversible oxidation of hydrosulphuric groups of essential enzymes, and such a group is not present in PPO. Further to this, there is no direct evidence that PPO activity declines. Fukumoto et al (2002) noted that efficacy of washing in water at 4°C was "significantly enhanced by chlorination" with 100 µg/ml scoring approximately less than half the discolouration observed in unchlorinated wash water in both photosynthetic and vascular tissues. Phenolic levels were found to be higher in photosynthetic than vascular tissue, whilst cut edge browning was less intense, which implies PPO activity is not an exclusively dominant factor in the resulting discolouration. Therefore, it may be that the cause of reduced pinking due to chlorination may be due to an effect on an alternative mechanism to PPO. Baur et al. (2005) noted that chlorination in cold wash water of 4 °C resulted in minor reductions in phenyalanine ammonia-lysase (PAL) enzyme, suggesting that inhibition of other parts of the pigmentation pathway may be involved.

There has recently been a drive towards the use of lower chlorine concentrations. However, in industrial practices the use of 20 mg L^{-1} of free chlorine has resulted in the presence of microbes (Luo et al., 2018), likely due to the poor regulation of wash water pH (when the pH rises the proportion of free chlorine in the active form of hypochlorous acid decreases). Currently we understand the UK industry still practises applications ranging from 50 to 80 mg L^{-1} free chlorine with a two-minute exposure time. UC Davis recommend 100 to 150 mg L^{-1} total chlorine, to ensure an adequate level of free chlorine is available for activity (Suslow, 1997). The 100 mg L-1 total chlorine concentration that resulted in the least pinking in Study A, and 200 mg L-1 in Study B, equate to approximately 45 mg L-1, and 90 mg L-1 of free chlorine respectively. These figures are within the range currently stipulated and Study A suggests suppliers could safely move towards the trend in Europe to reduce chlorine levels in the wash water of fresh produce whilst maintaining lettuce of high sensory quality.

Study B further showed that where chlorine washing is applied lettuce stored in MA packaging could be potentially supplied with oxygen at the higher end of the recommended spectrum, perhaps as high as 6% initial oxygen. This would allow for longer shelf lives as 'off odours' and other quality issues arising from the initiation of fermentation will not be induced by such a low initial oxygen content as early in the shelf life.

Conclusion

Chlorine concentrations in wash water of lettuce impact on the incidence and intensity of pinking exhibited in cut edge lettuce packaged in air and packaged in MAP with 6% initial oxygen. The use of specific chlorine content in the wash water of lettuce bagged using passive MA could significantly reduce the level of pinking observed over shelf life and in active MA with initial oxygen concentration of >6%. Regular testing to ensure the total chlorine concentration in wash water is maintained over 100 mg L^{-1}, alongside effective

packaging practices should allow processors to reduce the likelihood and severity of pinking whilst making no additional changes to their running costs or production line equipment.

Further investigations on concentration effects in the range of 100 mg L^{-1} total chlorine and investigations into the impact of chemicals of similar composition to trocolsene sodium (used in study A), and sodium hypochlorite (used in study B) on lettuce tissue components to identify the causes behind chlorine inhibition of cut edge pinking would be of value. This could lead to the identification of the optimum chlorine doses to limit pinking at different stages in the season when MAP alone is insufficient, and without additional costs in the production line.

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Table 1: Scoring descriptions for pink discolouration in processed lettuce

Score	Proportion of pinking	Intensity of pinking
0	Not present	
1	< 5 pieces	Very light pink (barely visible)
2	≤ ¼ pieces	Light pinking
3	≥ ¼ ≤½	Notable pinking (Unsaleable)
4	≥ ½ ≤ ¾	Red
5	≥ ¾	

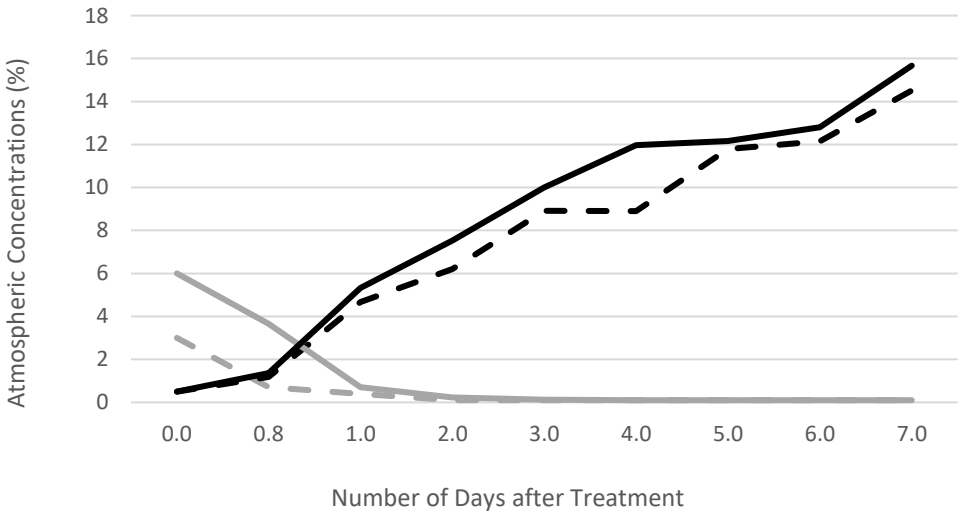

Figure 1: Concentrations of oxygen and carbon dioxide maintained in safepod™ chambers to simulate the atmosphere within modified atmosphere bags. Black lines indicate carbon dioxide concentration and grey lines indicate oxygen concentration. Broken lines indicate profiles for

303 bags with an initial oxygen concentration of 3% in the headspace of bag, unbroken lines are
304 profiles for bags with an initial oxygen concentration of 6% oxygen.

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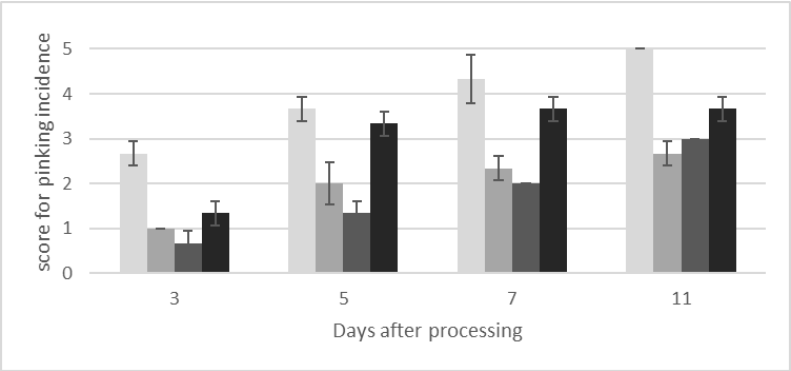
306 A.



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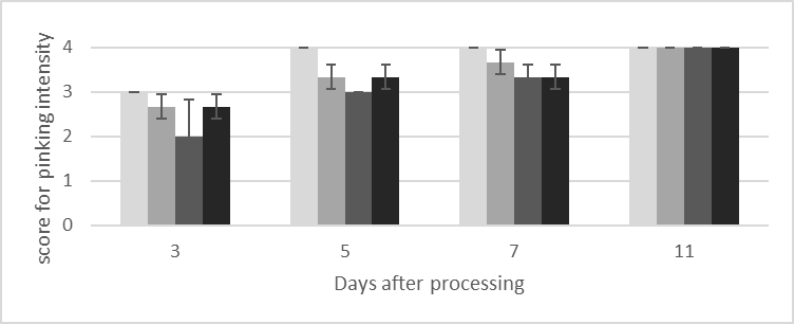
308 B.



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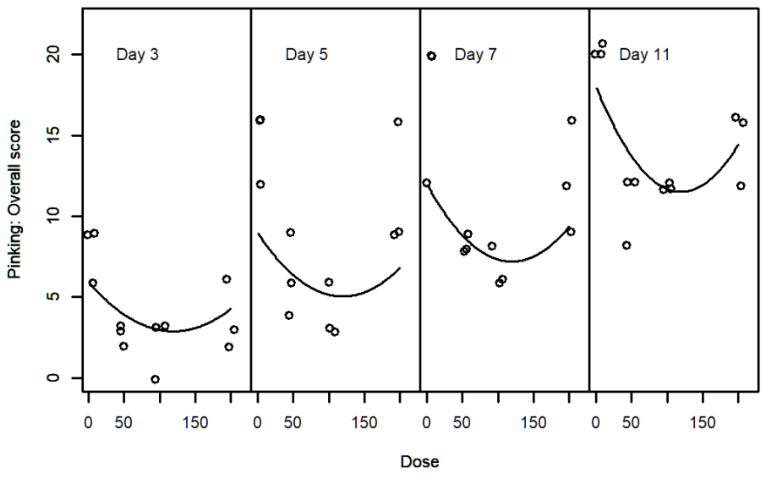

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312 Figure 2: (A) pinking incidence score, (B) pinking intensity score for shredded lettuce
313 subjected to four different chlorine wash water treatments and stored in air at 5°C for up to 11
314 days. Data are the mean of three replicates, error bars are standard error. (C) Results of multi
315 regression with quadratic curve for overall pinking score against chlorine dose. Individual

316 data points shown. Effects of day and quadratic dose term are both highly significant,

317 multiple regression anova. P value <0.0001.

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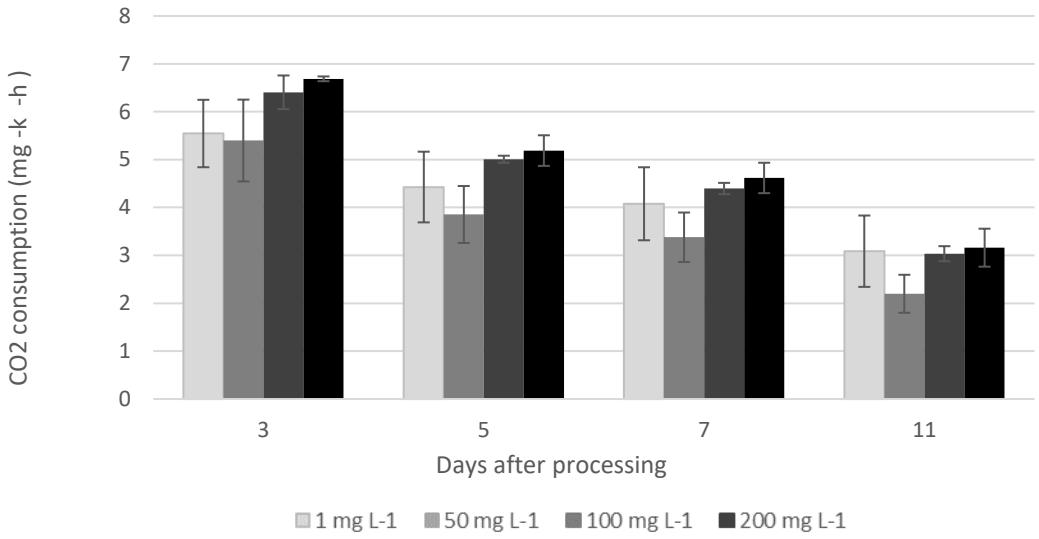


321

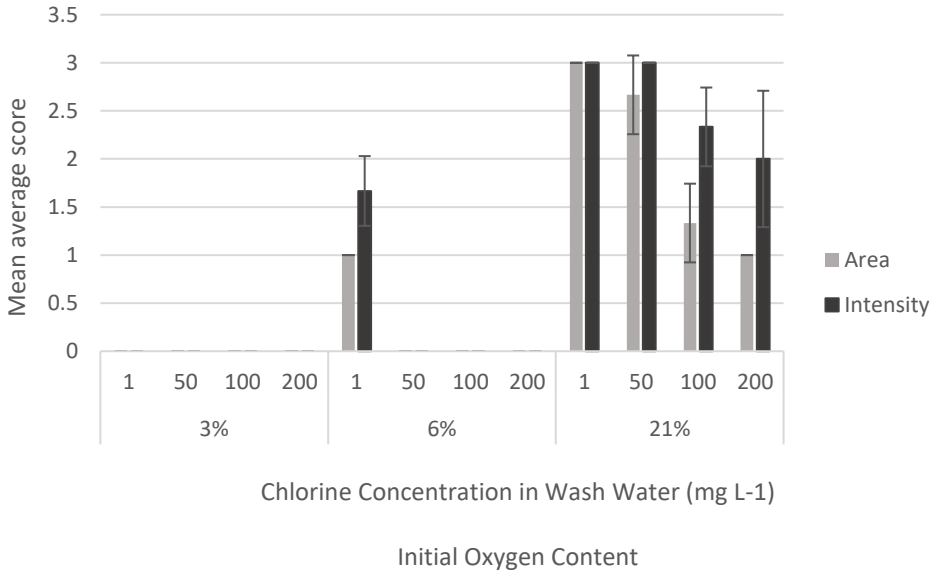
322 Figure 3: Respiration rates in lettuce washed in four different wash water chlorine

323 concentrations after shredding and stored in air for up to eleven days. The data are the mean

324 of three replicates per treatment, error bars are standard error.

325

326 A.



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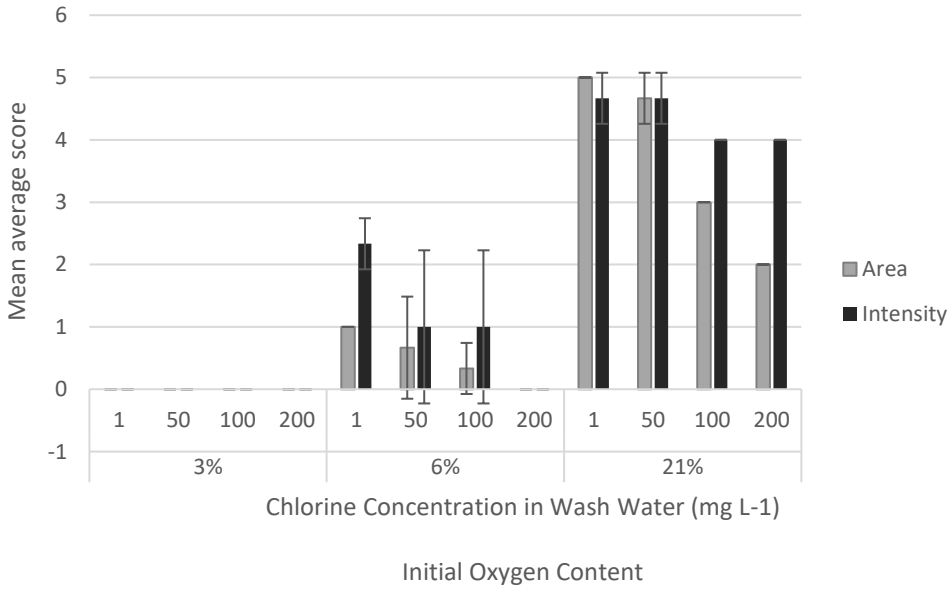
328 B.



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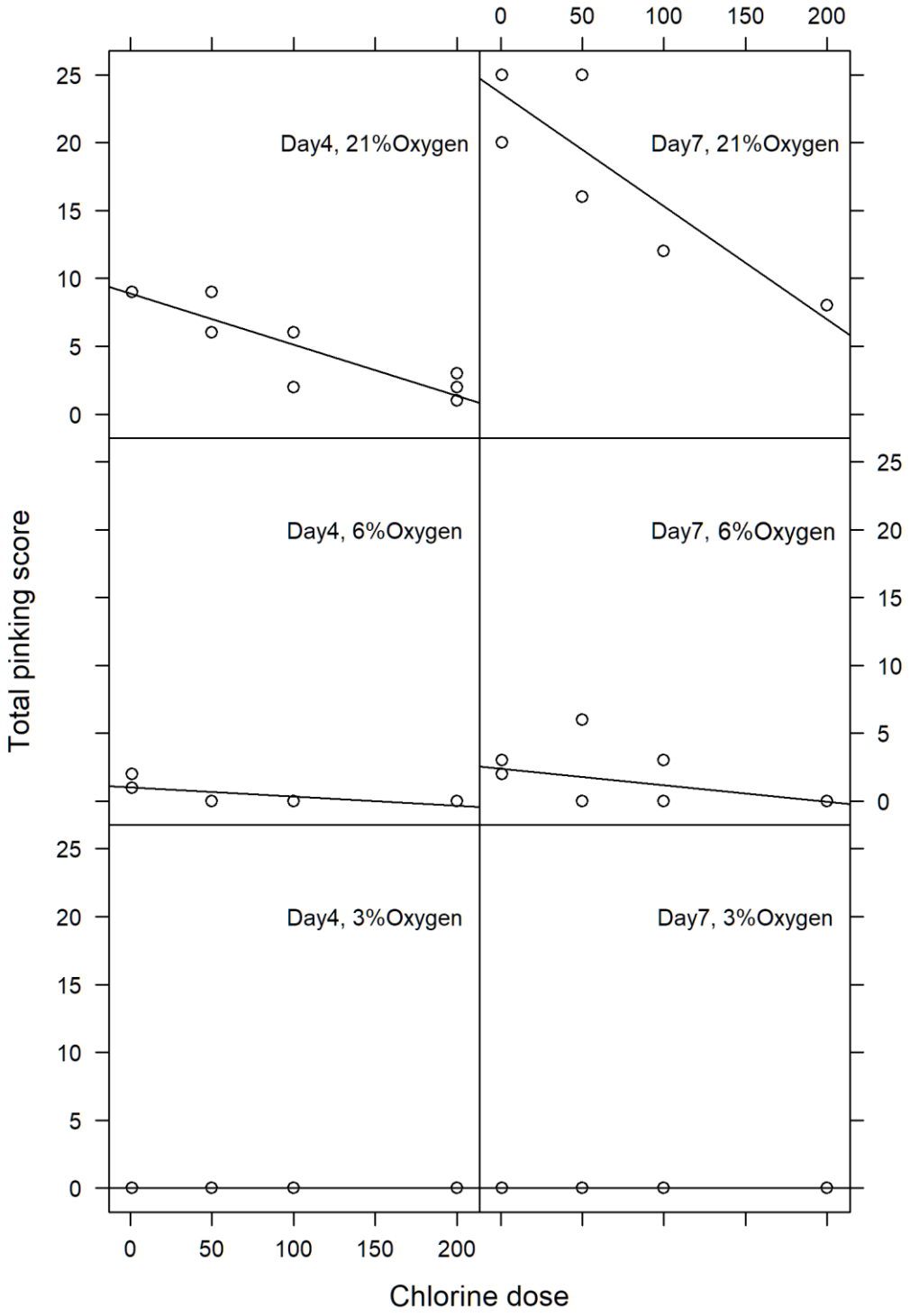

Figure 4: Pinking intensity and Incidence scores recorded in lettuce washed in increasing levels of chlorine concentration when stored in air, and in two 'bagged' profile atmospheres

335 with an initial oxygen content of 3% and 6%, 4 days (A) and 7 days (B) and after processing.
336 Error bars show standard error of three replicates per treatment. (C) Results of a linear
337 regression of overall pinking score against chlorine dose for each atmosphere profile and
338 assessment day. Effects of dose, oxygen profile, day of assessment are all highly significant
339 $P<0.001$. All interactions are significant to $P<0.01$.